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Interferon-γ inducible protein-10 and interleukin 28B gene polymorphism as predictive markers for genotype 4 hepatitis C virus treatment response

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Abstract. HCV genotype 4 dominates the HCV epidemic in Egypt. Drug resistance was the most serious side effect that reflects bad clinical outcome. Several studies had demonstrated that baseline serum interferon-γ-inducible-protein 10 (IP-10) levels and interleukin 28B polymorphisms were associated with the resistance to the standard of care pegylated interferon alpha and ribavirin (PEG-IFNα/RBV) therapy and development of post-treatment relapse. Our purpose was to assess the predictive value of combining IP-10 levels and IL28B genotypes to PEG-IFNα/RBV therapy response in Egyptian chronic HCV infection patients with genotype 4. Ninety Egyptian patients chronically infected by HCV genotype-4 treated with pegylated interferon alpha and ribavirin (PEG-IFNα/RBV) therapy were enrolled. Serum IP-10 levels were determined by enzyme linked immunosorbent assay pre- and post- treatment. IL-28B (rs12979860 and rs8099917) polymorphisms were performed by PCR-RFLP in all patients. Overall, 38 patients (42.2%) achieved sustained virologic response (SVR) and 52 (57.8%) patients have non-viral response (NVR). Pretreatment serum IP-10 mean levels were significantly lower in patients who achieved SVR than in NVR ($P<0.05$). CC genotype in IL-28B polymorphism (rs12979860) was the favorable genotype as 65.8% achieved SVR, while TT genotype in IL-28B polymorphism (rs8099917) was the favorable genotype as 81.5% achieved SVR. Baseline IP-10 was significantly correlated to genotypes CC in rs12979860 and TT in rs8099917. Combined use of serum baseline IP-10 levels with IL-28B polymorphisms could improve the prediction of SVR to PEG-IFNα/RBV therapy in Egyptian chronic HCV infection patients with genotype 4.

INTRODUCTION

Hepatitis C Virus (HCV) causes viral hepatitis infection that is a major global health challenge (WHO, 2015); Egypt has the highest world prevalence of hepatitis C virus (HCV) infection, which is associated with substantial disease and economic burden (El Zanaty *et al.*, 2019). HCV genotype 4 distribution is restricted to Egypt, Central Africa, and the Middle East regions. Genotype 4 (subtype 4a in particular) dominates the HCV epidemic in Egypt (Messina *et al.*, 2015). In 2015, the Egyptian Health Issues Survey (EHIS) was done to re-estimate the prevalence of HCV infection in Egypt. Among those aged 15-59 years, there was a significant reduction in prevalence of HCV antibody 14.7 to 10.0%, and HCV RNA from 9.9 to 7.0% (Kandeel *et al.*, 2017). The combined remedy of pegylated interferon alpha; acts as antiviral and immunoregulatory cytokine; and ribavirin; an antiviral prodrug that interferes with RNA metabolism; was denoted as the existing standard of care in chronic HCV genotype 4 (Varghese *et al.*, 2009).

Successful treatment, termed sustained virologic response (SVR), is defined as undetectable HCV RNA after six months of

cessation of treatment (Okita *et al*., 2014; Sato *et al*., 2014). Unfortunately, this therapy is expensive and the extended peginterferon and ribavirin therapy often accompanied with serious side effects (Sharma, 2010). Drug resistance is the most serious side effect that reflects bad clinical outcome (Dzekova-Vidimliski *et al*., 2015). Many studies have spotted several factors that related with the resistance to PEG-IFNα/RBV therapy and development of post-treatment decline (Tsubota *et al*., 2011). These factors include age, gender, obesity, HCV genotypes, viral load, Interleukin 28B single nucleotide polymorphisms (El-Khazragy *et al*., 2017).

In 2009, genome-wide association studies have shown that single nucleotide polymorphisms (SNPs) near the IL28B gene on chromosome 19 were powerfully linked to viral response and outcomes of treatment in HCV infected patients. Two SNPs are described to be highly predictive of a favorable treatment response: rs2979860 and rs8099917. They were found to be strongly predictive of treatment response with CC (rs12979860) and TT (rs8099917) genotypes (Firdaus *et al*., 2014). However, the study was limited to HCV genotype 3.

Interferon λ inducible protein 10 (IP-10) is a small cytokine that belongs to CXC chemokine family and known as C-X-C motif chemokine 10 (CXCL10). It is a 10 kDa protein, encoded by the CXCL10 gene (Neville *et al*., 1997). IP-10 is secreted in response to IFN-α by several cell types; include endothelial cells, monocytes, and fibroblasts (Luster *et al*., 1985). Several studies have shown that the IP-10 may act as a prognostic marker for HCV treatment outcome (Romero *et al*., 2006). Studies found that the baseline pre-treatment plasma levels of IP-10 are elevated in chronic HCV infected patients of genotypes 1 who do not achieve a sustained viral response (SVR) after completion of antiviral treatment (Lagging *et al*., 2006). Similarly, a relatively robust association between low baseline plasma IP-10 levels and a favorable viral kinetic response was distinguished during combined treatment with PEG-IFNα/RBV in HCV infected patients with genotypes 1 and 4. Thus, low circulating IP-10 levels before the onset of treatment were associated with a promising early decrease in serum viral loads, along with a sustained elimination of the virus after the completion of treatment (Romero *et al*., 2006).

Better predictors of SVR would assist in recognizing patients who should response before the beginning of the combined therapy with PEG-IFNα/RBV. We aimed to assess the association of pretreatment IP-10 levels with IL28B SNPs rs2979860 and rs8099917 as predictors of treatment response to PEG-IFNα/RBV in Egyptian chronic HCV infected patients with genotype 4.

SUBJECTS AND METHODS

Patients

This cross-sectional study enrolled 90 HCV chronic infected Egyptians patients from Ain Shams University Hospitals, after taking the approval of the research ethics committee of Faculty of Medicine, Ain Shams University which is in accordance with the Helsinki Declaration. All patients have signed an informed consent. The patients were selected according to specified inclusion and exclusion criteria.

The inclusion criteria: detection of HCV viremia proved by HCV-RNA level of 5.0 log IU/ml determined by the COBAS TaqMan HCV test, confirmed HCV genotype 4 and previously untreated. Patients chronically infected with hepatitis B virus or human immuno- deficiency virus, or with other liver disease such as autoimmune hepatitis and primary biliary cirrhosis as well as with HCC were excluded from this study.

Antiviral therapy and evaluation

All chronic patients included in the study were genotype 4 and were all subjected to pegylated interferon-alfa 2a 180 mcg per week or pegylated interferon-alfa 2b 1.5mcg per kg body weight in combination with ribavirin 600-1400 mg per day according to body weight for 24-48 weeks. Patients were assessed initially at diagnosis (untreated) and 24 weeks after therapy. Patients were subdivided according to their response for PEG-IFNα/RBV combined therapy into two

groups; sustained viral response group (SVR): those who showed undetectable HCV baseline viremia by the end of the treatment protocol 24 weeks post therapy. Null viral response group (NVR): those who achieved, stability or decrease less than 2 folds in baseline HCV viremia at 12 weeks post therapy (Shiffman, 2006).

Laboratory assessment

All procedures performed in this study involving human participants were in accordance with the ethical standards of Ain Shams University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Before treatment, laboratory investigations collected from the patient's clinical sheets including; Complete blood count (CBC) analysis done by Coulter counter (Coulter JT 660, Coulter S Ltd, UK) and Laboratory assays including: liver transaminases; ALT and AST, serum bilirubin, albumin and creatinine were determined by an automatic biochemical analyzer.

IL28B Single Nucleotide Polymorphism

Two ml of blood samples was collected in an EDTA containing tube for DNA extraction used in genotyping of IL28B polymorphism. DNA was extracted from whole blood using QIAamp DNA blood mini kit (QIAGEN, Valencia, CA) and quantitated on the NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific). After quantification of DNA by UV spectrophotometer, 100ng of genomic DNA was used for each 20 µl PCR reaction (Newton *et al*., 1989).

The rs12979860 and rs8099917 SNPs genotyping was carried out by polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP). For rs12979860, oligonucleotide primers were: 5'- AGG GCC CCT AAC CTC TGC ACA GTC T -3' (sense), and 5'- GCT GAG GGA CCG CTA CGT AAG TCA CC -3' (antisense). For rs8099917, oligonucleotide primers were: 5'- TTC ACC ATC CTC CTC TCA TCC CTC AT -3' (sense) and 5'- TCC TAA ATT GAC GGG CCA TCT GTT TC -3' (antisense).

PCR reaction conditions was done in (30 µl) containing approximately 250 ng DNA with 0.25 µM of both primers, 0.1 mM of each dNTP, 1 x PCR buffer, 1.5 mM MgCI2 and 1 U Taq polymerase (Promega, Madison, WI, USA). The cycling conditions were: initial denaturation at 94°C for 10 min, followed by 40 cycles of: denaturation at 94°C for 1 min, annealing at 60°C for 40 s, and extension at 72°C for 1 min.

In order to perform RFLP assay for the rs12979860 genotype, 20 µl of amplicons were digested with 5U of BstU I restriction endonuclease (New England Biolabs, MA, United States) at 60°C for 2 h. BstU I digestion of allele CC yields fragments of 184, 105, 89 and 25 base pairs, whereas DNA containing the allele TT polymorphism yields fragments of 184, 130 and 89 base pairs. In RFLP assay for the rs8099917 genotype, 30 µl of reagent mixture the amplicons were digested with 1U of 1BseMI (BsrDI) restriction endonuclease (Thermoscientific, United States) at 55°C for 2 h. BsrDI digestion of allele TT yields one fragment of 401 base pairs, whereas DNA containing the allele GG polymorphism yields fragments of 150 and 280 base pairs. Restriction digestion products for each were separated on agarose gels stained with ethidium bromide for visualization on a UV trans-illuminator (Venegas *et al*., 2011) (Figure 1 & 2).

Serum IP-10 measurements

Serum IP-10 concentrations were measured in samples collected at baseline by using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Sunred, Shanghai, China). All blood samples were stored at -20°C till used.

Data analysis

Statistical analysis package for SPSS (statistical package for social science) Statistics version 22. Quantitative variables were summarized using Mean ± SD. Categorical variables were compared using the $\chi 2$ test and expressed as actual numbers and their percentages. $P < 0.05$ were considered significant. The two-tailed test.

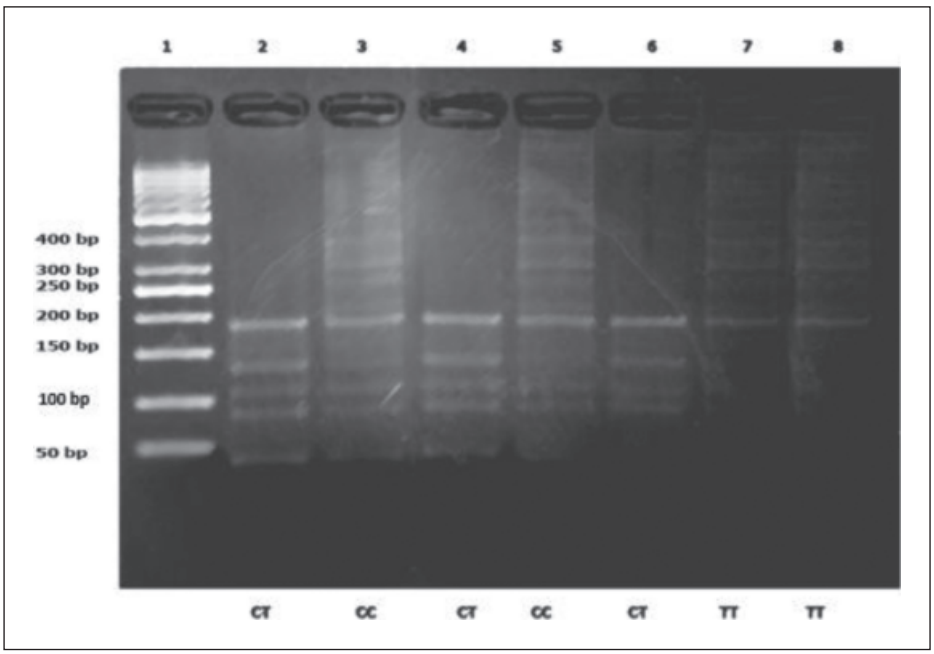

Figure 1. RFLP of the amplified product of rs12979860 digested with BstU I enzyme among the HCV patient groups: lane 1, 50-bp DNA ladder; lanes 2,4,6 the electrophoresis pattern of genotype CT; lanes 3,5 restricted PCR product of genotype CC; lanes 7,8 restricted PCR product of genotype TT.

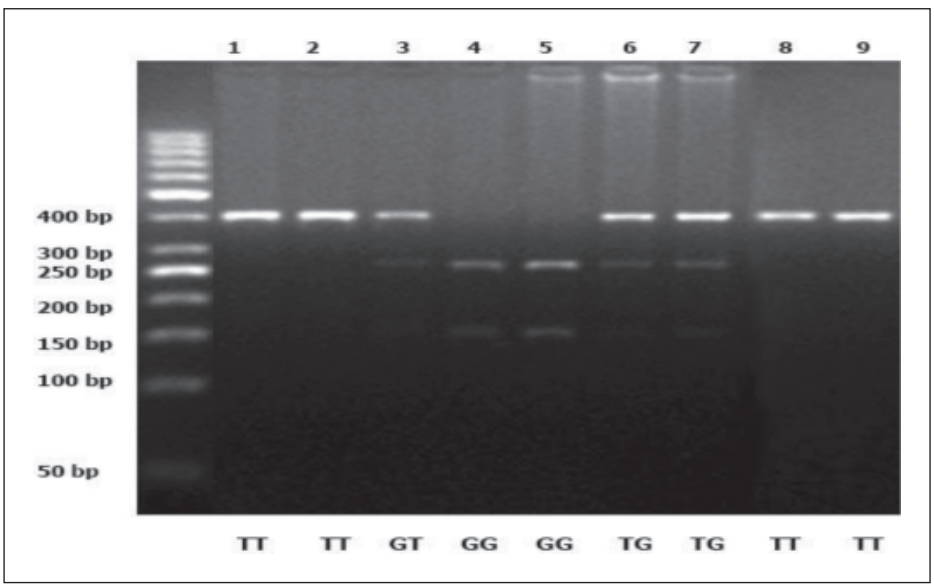

Figure 2. RFLP of the amplified product of rs8099917 digested with BseMI (BsrDI) enzyme among the HCV patient groups: first lane, 50-bp DNA ladder; lanes 1,2,8,9, genotype TT; lanes 3,6,7 genotype TG; lanes 4,5 genotype GG.

RESULTS

Patients characteristics

The demographic and laboratory characteristics of the studied patients enrolled in this study are presented in Table 1.

Impact of IL28B polymorphism and serum IP-10 levels on treatment outcomes

For IL28B (rs12979860), most patients were heterozygous CT genotype (n=47) followed by the homozygous CC (n=34) then the TT genotyped case (n=9). While for IL28B (rs8099917); the homozygous TT genotype was the predominant genotype (n=40) followed by GT genotype (n=38) then GG genotype (n=12).

The frequency of IL28B SNPs showed that, for rs12979860; CC was the favorable genotype that achieved significantly higher SVR rates (65.8%) compared to CT and TT genotypes (28.9% and 5.3% respectively). Regarding genotype rs8099917, TT was the favorable genotype that achieved significantly higher SVR rates (81.5%) compared to GT and GG genotypes (13.2% and 5.2% respectively) (P<0.05). HCV viral load is a significant discriminator between SVR and NVR patients (p<0.05) (Table 2).

Moreover, the comparison between the different IL28B (rs12979860) genotypes, showed a significant difference in mean values of ALT and AST levels (p<0.05) in CC genotype as compared to CT and TT genotypes. While regarding IL28B (rs8099917) genotypes, the ALT, AST, albumin and bilirubin levels were significantly different in TT genotype as compared to GT and GG (p<0.05). No significant difference was detected among other parameters (Table 3).

Mean IP-10 levels at baseline were significantly lower in patients who achieved SVR than in those with NVR (SVR, 331.7±44.4 pg/mL vs NVR, 642.6±114 pg/ml; p<0.05). No significant difference between mean IP-10 levels at baseline and post therapy although there was slight decrease in IP-10 level in SVR and a slight increase in NVR (Table 4).

Comparing IP-10 levels with different clinico-pathological parameters revealed a statistical significance difference in (AST, ALT, albumin and bilirubin) (p<0.05) (Table 5).

Association of IL28B genotypes and baseline serum IP-10 levels with treatment response

In 90 patients, the predictive value of the combination of IP-10 levels with IL28B genotypes was significant with SVR rates. Both baseline IP-10 and post therapy were significantly associated with interleukin 28B rs12979860 genotypes. Lower baseline IP-10 was significantly correlated to homozygous CC genotype with significant lower values while its level was higher in homozygous TT and heterozygous CT (P<0.05). Also, both baseline IP-10 and post therapy were significantly associated to interleukin 28B rs8099917 genotypes. Lower baseline IP-10 was significantly correlated to homozygous TT genotype with significant lower values while its level was higher in homozygous GG and heterozygous GT (P<0.05) (Table 6).

DISCUSSION

HCV genotype 4 distribution is restricted to Egypt, Central Africa, and the Middle East regions. Genotype 4 (subtype 4a in particular) dominates the HCV epidemic in Egypt (Messina *et al.*, 2015). The combined therapy of PEG-IFNα/RBV represented the existing standard of care in chronic HCV genotype 4 (Varghese *et al.*, 2009). Unfortunately, this therapy is expensive and the continued PEG-IFNα/RBV therapy often associated with serious side eects (Sharma, 2010). Drug resistance was the most serious side effect that reflects bad clinical outcome (Dzekova-Vidimliski *et al.*, 2015).

Numerous studies tried to examine the common causes that lead to the resistance to PEG-IFNα/RBV therapy. Former study conducted by our research team found a great linkage between individuals' response to therapy and the genotypic variation among individuals (El-Khazragy *et al.*, 2019).

The current study was planned to assess the predictive value of joining IP-10

levels and IL28B genotypes to expect PEG-IFNα/RBV therapy response in chronic HCV infection patients with genotype 4.

IL-28B has a role in the regulation of intracellular interferon stimulated gene (ISG) expression (Sheppard *et al.*, 2003). IL-28B exhibits antiviral activity, having an influence on natural clearance of HCV (Par *et al.*, 2011).

In the present study, CT genotype of IL-28B (rs12979860) was the most common in the studied patients (52.2%) while CC genotype identified in 37.7% of patients and TT was detected in 10% of patients. Compatible with us Bakr *et al.* (2015) noticed that, the frequencies of the IL-28B (rs12979860) was common in CT followed by CC then TT genotypes. Conversely, Asselah *et al.* (2012) and EL-Awady *et al.* (2012) found that CC was most common in their studies.

Six months after termination of therapy, 52 (57.8%) patients achieved SVR and 38 (42.2%%) patients showed NVR. The frequency of CC genotype of IL-28B (rs12979860) who achieved SVR was 65.8%, which is significantly higher compared to CT (28.9%) and TT (5.3%) genotypes. Bakr *et al.* (2015) reported that, 84.4% of patients with the CC genotype achieved SVR, compared to 45.5% of CT and 23.3% of TT genotypes. Thus, CC genotype was the most favorable genotype for treatment.

Furthermore, our results revealed that, TT genotype of IL-28B (rs8099917) was the most common in the studied patients (44.0%) while GT genotype identified in 42% and GG was detected in 14.0%. In agreement with our study, Ragheb *et al.* (2014) and Esmail *et al.* (2016) found that TT genotype was the most common among their studied patients.

In our study, also there was a significant difference between various IL-28B (rs8099917) genotypes in response to therapy. SVR was detected in 81.5% of patients with homozygous TT (rs8099917), while SVR represented 13.2% of heterozygous GT and 5.2% of the GG genotype patients. Similarly, Ragheb *et al.* (2014) suggested that the 65.7% of TT genotype (rs8099917) achieved SVR, which is significantly higher compared with GT 7.7%, although NVR was observed in GG genotype. Thus, TT genotype was the most favorable genotype.

The meticulous biological pathways emphasizing the IL-28B gene SNP association with treatment response and viral clearance remain mysterious. Studies investigated IL28B polymorphism revealed that IL-28B polymorphism variants genotypes are linked with endogenous activation of host innate immunity (Sultana *et al.*, 2016). As host innate immune mechanisms including IFN-γ control viral infection (Ibrahim *et al.*, 2013). The binding of IFN-γ to its receptor is followed by several activation reactions that end with the induction of ISGs (Marcello *et al.*, 2006), which suppresses viral infection.

IP-10 is an IFN inducible chemokine that binds to chemokine receptor, CXCR3, on lymphoid cells for driving them to inflammatory sites (Thomas *et al.*, 2009). It has been shown that CXCR3 ligands were elevated in livers and sera of CHC patients. IP-10 secreted by monocytes, and fibroblasts in response to IFN-α was correlated with treatment responses (Helbig *et al.*, 2004).

In the current study, we observed that baseline IP-10 was significantly correlated to HCV response to therapy where lower mean levels of IP-10 was significantly correlated to SVR with a significantly lower value 331.7 pg/ml compared to 642.6 pg/ml in the non-responder patients ($P<0.05$) but with no significant difference between baseline and post treatment. Also, we proved that diminished baseline serum IP-10 levels are correlated with SVR in HCV genotype 4 infected patients treated with PEG-IFNα/RBV therapy. Several previous studies reported the relation between IP-10 levels and a response to anti-HCV therapy (Harvey *et al.*, 2003; Lagging *et al.*, 2006; Romero *et al.*, 2006; Fattovich *et al.*, 2011). Lagging *et al.* (2006) reported that low pretreatment IP-10 levels predict SVR in patients infected with the HCV genotype 1.

Our results reveled that after effective PEG-IFNα/RBV therapy with SVR, concentrations of serum IP-10 decreased to levels lower than baseline while they were slightly increased or unchanged in NVR, suggesting that HCV itself may be responsible for

elevated IP-10 concentrations found in HCV infected patients. In the present study, we recorded that patients with lower baseline IP-10 level were much more likely to achieve SVR and favorable outcome response to PEG-IFNα/RBV therapy in HCV infected patients.

In the existing study, both baseline and post therapy IP-10 were significantly correlated to IL28B rs12979860 and rs8099917 genotypes. Lower baseline IP-10 was significantly correlated to homozygous carriers of the favorable CC (rs12979860) and TT (rs8099917), as compared with patients carrying unfavorable genotype. As known, baseline hepatic ISGs levels significantly affect the outcome responses of IFN based treatment in HCV infected patients.

Therefore, if we combined baseline IP-10 with IL28B of SNPs rs12979860 and rs8099917, the prediction of SVR could become better than if using one of them alone as a predictive factor, it could improve the prediction of SVR in favorable allele carriers of IL28B, rs12979860 CC and rs8099917 TT.

In the current study there was significance difference ($p<0.05$) observed between different IL28B rs12979860 genotypes with ALT and AST. In contrast, there were no significant difference in other parameters (Albumin, Bilirubin, creatinine, Hb, TLC and platelet) ($P>0.05$). Hendy *et al.* (2011) detected highly significant difference between IL28B rs12979860 genotypes with ALT and AST levels.

In IL28B rs8099917 genotypes there was a significant difference ($p<0.05$) with liver function tests (ALT, AST, Albumin and Bilirubin) while no significant relation was found with creatinine and the hematologic parameters ($P>0.05$). Similar result was reported with Hendy *et al.* regarding AST and ALT levels. On the other hand, Esmail *et al.* (2016) detected no significant difference of ALT and AST with IL28B rs8099917 genotypes.

In the current study, a significant relation ($p<0.05$) was observed between IP-10 levels and liver function tests (ALT, AST, Alb and bilirubin). In contrast, there were no significant relation with creatinine and hematologic parameters ($P>0.05$). In disagreement with our study, Omran *et al.* (2014) detected no significant difference between IP-10 levels and other biological parameters.

Previous study has targeted the use of IP-10 and IL28B SNPs in prediction of treatment outcome of HCV but in HCV with genotype 1 (Zhang *et al.*, 2016), but no studies targeted them in prediction of treatment outcome of HCV genotype 4; which is endemic in Egypt.

In conclusion, the combined use of serum baseline IP-10 levels with IL-28B polymorphisms could improve the prediction of SVR to PEG-IFNα/RBV therapy in Egyptian chronic HCV infected patients with genotype 4. Also, the prediction of response helps to identify patients who are likely or unlikely to achieve SVR and thus reduce high cost and the risk of side effects of therapy.

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Conflict of interests

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